

AN ABSTRACT OF THE THESIS OF

Katherine E. Chiavarini for the degree of Masters in Science in Zoology presented on May 29, 1997. Title: Rapid Effects of Corticosterone on Stress-Related Behaviors in an Amphibian.

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Frank L. Moore

In the wild, when an animal is exposed to predators or harsh conditions, the stress response is often associated with fleeing behaviors, which are seen as increased locomotor behavior. Handling-stress procedures and intracerebroventricular (icv) injection of corticotropin-releasing factor (CRF) have both been shown to cause an increase in locomotor activity in roughskin newts (*Taricha granulosa*). The present experiments were designed to determine if icv administration of corticosterone (CORT) prevents stress-induced locomotor increases in activity, if it prevents CRF-induced increases in locomotor activity, and if the time-course and pharmacological specificity of the CORT effects on locomotor activity fit the model for intracellular or membrane receptors.

In experiment 1, newts which had been injected with CORT or dexamethasone (DEX) received a standardized handling-stress procedure. Corticosterone administration was able to suppress the increase in locomotor activity in newts exposed to handling-stress at 20 minutes after administration. This effect was transient (no longer present at 2 hours after the injection) and not mimicked by DEX, a synthetic glucocorticoid that binds

to intracellular and not membrane receptors. In experiments 2 and 3, either CORT or DEX was administered in the same icv injection with CRF. CORT suppressed CRF-induced locomotor activity in some cases, but this action of CORT seems to be context dependent. Results for DEX-injected newts were confounded the failure of CRF to induced significant increases in locomotor activity. There was variability in the effect of CRF on locomotor activity across seasons. Based on time-course and specificity, it appears that CORT can modulate locomotor activity in newts through mechanisms involving the membrane receptor. Variability in the effects of CRF on locomotor activity in newts suggests there may be seasonal differences in responses to stress.

Rapid Effects of Corticosterone on Stress-Related Behaviors in an Amphibian

by

Katherine E. Chiavarini

A THESIS

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degree of

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Redacted for Privacy

Major Professor, representing Zoology

Redacted for Privacy

Chair of Department of Zoology

Redacted for Privacy

Dean of Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorized release of my thesis to any reader upon request.

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Katherine E. Chiavarini, Author

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First and foremost I would like to give special thanks to my advisor Frank Moore.

The freedom to explore and investigate avenues which were most interesting to me afforded me the ability to mature academically. In addition, his willingness to take time out of an extremely busy schedule whenever I needed advice, guidance, or help with revisions was indispensable, especially during the rapid pace of finishing up my thesis.

I thank Rick Thompson for advice, encouragement, and empathy in both academic and nonacademic realms. I thank Jill DeVito for always being there as a friend and a colleague. I also want to express my appreciation to the zoology faculty and graduate students for all the help, advice and friendship which they provided to me.

I also thank the statistics department for providing free guidance in my statistical analysis. Specifically, I thank Teri Utlaut and Cliff Pereira for providing special attention to my project and patiently learning enough about CRF, CORT, newts, and my experimental design to help so thoroughly.

I thank my housemates, Aaron and Troy for being great friends and also for allowing for 'give and take' with chores, especially when things got hectic. I thank my newly found friend and confidant, Lavanya Muregesan for always brightening even the darkest moments with her generosity, warmth, and caring. I also thank my dear friend Monica Price, for her energy as well as her strong spirit and character which always serve as source of strength and comfort for me.

Most importantly, I want to give special thanks to my parents for always encouraging me to be independent, regardless of whether that meant they would see less of me. I also thank them for all of the support, encouragement, understanding, confidence, and guidance they always provide me, no matter what my choices are.

TABLE OF CONTENTS

	<u>Page</u>
INTRODUCTION	1
EXPERIMENTAL METHODS	12
Study animals	12
Injection Procedures	12
Testing arena	13
Behavioral Tests	14
Statistical Analysis	15
RESULTS AND SPECIFIC METHODS	16
Experiment 1	16
Experiment 2	22
Experiment 3	30
DISCUSSION	34
BIBLIOGRAPHY	45
APPENDICES	52
Appendix A CRF and CORT: time course studies	53
Background	53
Experiment A.1	53
Methods and Rationale	53
Results and Discussion	54
Experiment A.2	59
Methods and Rationale	59
Results and Discussion	59
Appendix B CRF and Fluoxetine interaction studies	62
Background	62
Methods and Rationale	62
Results and Discussion	63

LIST OF FIGURES

<u>Figure</u>		<u>Page</u>
1.	The effect of CORT administration on handling stress-induced locomotor activity of male roughskin newts.....	17
2.	The effect of DEX administration on handling stress-induced locomotor activity of male roughskin newts.....	19
3.	Effects of CORT on CRF-induced locomotor activity in male roughskin newts	24
4.	Effects of CORT on CRF-induced locomotor activity in male roughskin newts	26
5.	Effects of DEX on CRF-induced locomotor activity in male roughskin newts	28
6.	Effects of CORT or DEX on CRF-induced locomotor activity in male roughskin newts	32

LIST OF APPENDIX FIGURES

Figure	Page
A.1a Effects of CORT (ip) on CRF-induced locomotor activity in male roughskin newts. CORT given 5 minutes following CRF administration (replicate #1)	55
A.1b Effects of CORT (ip) on CRF-induced locomotor activity in male roughskin newts. CORT given 5 minutes following CRF administration (replicate #2)	57
A.2 Effects of CORT (ip) on CRF-induced locomotor activity in male roughskin newts. CORT given 5 minutes prior to CRF administration	62
B.1 Effects of fluoxetine (or anandamide)(icv) and CORT (ip) on locomotor activity in male roughskin newts	64
B.2 Effects of fluoxetine (or anandamide)(icv) and CORT (ip) on locomotor activity in male roughskin newts (dose-response, doses below 25 µg / 0.1 ml)	66
B.3 Effects of fluoxetine (or anandamide)(icv) and CORT (ip) on locomotor activity in male roughskin newts (dose-response, doses above 25 µg / 0.1 ml)	68

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Rapid Effects of Corticosterone on Stress Related Behaviors in an Amphibian

INTRODUCTION

An animal faces many stresses throughout its life and even throughout the duration of a single day. Potential prey have to be ready to respond quickly to the surprise appearance of a predator. A predator may need to have the ability to exert the extra spurt of energy required to catch and kill its prey even under harsh environmental conditions. Animals must be able to allow for large energy bursts to avoid predators or catch prey, sometimes for extended durations or distances. In other cases, animals may have to adjust their normal homeostasis to deal with harsh environmental conditions. They need to be able to adjust energy expenditures to supply the muscles with extra energy during bursts, even in times of near starvation, or to conserve energy under harsh conditions. In still other instances, they may have to limit their attack on pathogens or parasites to avoid immune and inflammatory responses from leading to excessive tissue damage (Bamberger et al. 1996). In fact, Chrousos et al. (1992) suggest that “ [human] physiological mechanisms for coping with adversity have not evolved appreciably over the past several thousand years” so that our “physiological responses to social pressures, information overload, and rapid change resemble those set into motion during physical danger and outright threats to survival.” (Chrousos et al. 1992).

Many facets of the neuroendocrine system are activated during the stress

response. First, corticotropin-releasing factor (CRF) is released within the brain and from neurons in the median eminence of the hypothalamus. Within the hypothalamo-pituitary axis, the nerve terminals in the median eminence secrete CRF which is transported to corticotropes within the adenohypophysis. CRF stimulates the secretion of corticotropin (ACTH) and β -endorphins. ACTH enters the general circulation and travels to the adrenal cortex (interrenal glands of amphibians, where it stimulates glucocorticoid secretion (mainly cortisol or corticosterone, depending on the species). The adrenal medulla also can be activated by stressful stimuli to secrete its hormones, epinephrine and norepinephrine. The stimulation of the adrenal medulla is controlled by neuronal pathways originating in the brain. CRF can also have direct actions in the brain, acting on specific target neurons within various brain regions known to respond to CRF. The large number of steps and pathways involved allows the body to fine-tune the stress response, since control can be exerted at any one of these steps.

CRF is involved in many components of the stress response. CRF has hypophysiotropic actions to regulate release of ACTH and β -endorphins and is secreted in response to intense physical or psychological stress (Merlo-Pich et al. 1993). Various stressors such as cold swim, physical restraint or ether inhalation in rats will cause an immediate release of CRF into the hypophysis and an increase in CRF synthesis in the paraventricular nucleus (PVN) (Chappell et al. 1986). Hypophysiotropic actions of CRF tend to be slow and typically elicit effects within hours or days of their onset.

CRF also regulates functions directly in the brain, including modifiable and

unlearned responses to stressors (Heinrichs et al. 1992). In response to various types of stressors (restraint, exercise, social defeat, or ethanol withdrawal) CRF can act directly in the brain to cause behaviors associated with stress. For example, microinjections of CRF, into the PVN, can enhance dose-dependent locomotor activity and “anxiogenic-like” effects in rats (Monnikes et al. 1992). Also in rats, intracerebroventricular (icv) administration produces dose-dependent behavioral activation of locomotor activity, rearing, and grooming (Sutton et al. 1982, Sherman and Kalin 1987). Administration of CRF antagonist during these tests blocks these behaviors, verifying that these actions are through CRF (Baldwin 1991; Heinrichs 1994). However, these behavioral effects of CRF are not blocked by hypophysectomy (Eaves et al. 1985) demonstrating that these actions are separate from hypophysiotropic actions. Neurotropic actions of CRF are typically rapid, occurring within seconds of CRF release or administration.

There are several distinct populations of CRF neurons in the brain. Many distinct brain regions which are shown to possess CRF neurons do not show changes in CRF concentration in response to stressors (Chappell et al. 1986). However, the locus ceruleus, Barrington’s nucleus and the olfactory bulb are specific brain regions which exhibit selective increases or decreases in CRF levels depending on the nature and duration of the stressor (Valentino et al. 1988; Imaki et al. 1991). In addition, CRF immunoreactivity has been shown in the PVN, the central nucleus of the amygdala, the bed nucleus of the stria terminalis, the substantia innominata, and the region of the locus ceruleus (Swanson et al 1983). The presence of CRF mRNA has been confirmed in most

of these regions using *in situ* hybridization techniques (Sawchenko 1987).

In roughskin newts (*Taricha granulosa*), CRF injection into the third ventricle of the brain has been shown to enhance locomotor activity (Moore et al. 1984, Lowry et al. 1990). Locomotor activity has been shown increase in response to CRF in a dose-dependent manner (Lowry et al. 1990). Hypophysectomy does not block these effects in newts so that, as in rats (Eaves et al. 1985), these CRF-induced behaviors appear to be independent of hypophysiotropic actions (Moore et al. 1984).

Lowry developed experimental procedures with newts in order to investigate the neuroendocrine responses to harsh or threatening conditions. He developed a standardized handling procedure which can cause a fairly consistent increase in locomotor activity (Lowry et al. 1990) which I also used in my experiments. The increase in locomotor activity following exposure to the handling-stress procedure was demonstrated to be prevented by the CRF antagonist, α -helical CRF (Lowry and Moore 1991). These results indicate that this handling-induced locomotion is a result of an increase in endogenous CRF in response to the stressor (Lowry & Moore 1991).

Corticosteroids, a type of steroid hormones that is associated with the stress response, help in the maintenance of homeostasis as well as in controlling physiological and behavioral changes associated with stress. Basal levels of corticosteroids produce general permissive effects on the animal's physiology. Corticosteroids achieve this by regulating membrane permeabilities (including transport mechanisms) and also by regulating protein synthesis, creating new enzymes or receptors (Norris 1997). The

permissive actions of corticosteroids have been shown to play a role in the action of the thyroid hormones, calcium-phosphorus balance, maintenance of muscle strength, glucose availability maintenance of normal functioning of the brain, maintenance of red blood cell formation, and many others (Asterita 1985, Norris 1997).

At higher concentrations, for example at levels found in a stressed animal, corticosteroids cause physiological changes that help the animal prepare for and survive the immediate perceived or actual threat to its well-being. Corticosteroids also suppress excessive defense reactions which could prove more detrimental to the animal than the threat itself. The actions of corticosteroids are pleiotropic, affecting changes in carbohydrate and mineral metabolism, the immune system, cardiovascular system, as well as behavioral changes (de Kloet et al. 1994).

Initially, under conditions of acute stress, corticosteroids decrease glucose uptake in some tissues, including adipocytes and some nerve cells. They also stimulate lipolysis in adipose tissue and the utilization of fats rather than glycogen and glucose for energy, thus conserving glucose and glycogen. Corticosteroids then begin to inhibit anabolism and promote catabolism of protein and catabolic effects on lymphoid, bone, connective and other body tissues. Through stabilization of lysosome breakdown and decrease in fibroblast activity, corticosteroids inhibit the inflammatory response. In addition, corticosteroids can prevent swelling by limiting the ability of white blood cells to reach the traumatized area and release more inflammatory substance with resultant loss of plasma to the tissues.

Chronic stress, when associated with persistently high levels of glucocorticoids, can lead to similar actions to those caused by acute stress which may have different effects because of the extended nature of these actions or can lead to totally different actions from those caused by acute stress. Among other effects, chronic release of corticosteroids can severely weaken the muscular system. Constant high levels of corticosteroids also suppress lymphoid tissues throughout the body, reducing the overall level of immunity to almost all foreign substances (Asterita 1985).

There is a wide range in the time course with which corticosteroids elicit their effects. In negative feedback actions of glucocorticoids, for example, there exist three phases: rapid feedback occurs within seconds to minutes of administration; intermediate feedback occurs within 2 - 10 hours; and delayed feedback occurs within days (Childs and Unabia 1990). Short-term effects of corticosterone have been demonstrated to inhibit CRF- and AVP-induced ACTH and β -endorphin release (Jones and Hillhouse 1976, Liu et al. 1995, Liu and Chen 1995), shorten the duration of ethanol-induced sleep in rats (Sze 1993), inhibit neuronal firing (Hua and Chen 1989, Rose et al. 1995) and inhibit sexual behaviors in newts (Moore and Miller 1985, Moore and Orchinik 1994). The long-term effects of chronically high concentrations of corticosterone have been shown to down regulate CRF receptors, inhibit CRF synthesis and release (Jones and Hillhouse 1976), and affect alertness and behaviors such as the further enhancement of CRF-enhanced acoustic startle reflex by corticosteroids which has been observed in rats (Lee et al. 1994).

There appear to be two main types of receptors for mediating corticosteroid action. Typically, intracellular receptors regulate genomic actions, and membrane receptors regulate non-genomic actions. Membrane receptors for steroids are becoming more widely recognized, with increasing evidence for membrane receptors for progesterone (Alexander et al. 1996, Baldi et al. 1995), aldosterone (Urbach et al. 1996), estrogens (Farhat et al. 1996), androgens (Gorkzynska 1995), and corticosteroids (Orchinik et al 1991).

There are two recognized types of intracellular corticosteroid receptors: type I and type II. Type I receptors are also called mineralocorticoid receptors because in mammals the naturally occurring ligand is the mineralocorticoid aldosterone. Type I receptors have high affinity for glucocorticoids and aldosterone (binding affinity: corticosterone = cortisol = aldosterone > dexamethasone: $K_d \sim 0.5$ nM for corticosterone in rats) (Reul and de Kloet 1985). With such high affinity, a high percentage of type I receptors are occupied at basal, non-stress levels of corticosterone in rats. Type I receptors appear to be involved in tonic, nonselective actions involved in maintaining homeostasis in most tissues. Type I receptors in the kidney regulate sodium ion reabsorption by binding aldosterone selectively. The type II (glucocorticoid) receptor has a tenfold lower affinity for corticosteroids than type I receptors (binding affinity: dexamethasone > cortisol > corticosterone > aldosterone: $K_d \sim 2.5-5$ nM for corticosterone values in rats). Therefore, in rats, the type II receptor is activated only during times when the animal has elevated levels of corticosteroids (Reul and de Kloet

1985). In this way, the type II receptor is able to display phasic actions involved in regulation of stress responses.

The traditional intracellular receptor model for steroid hormone actions involves gene transcription and protein synthesis as its mode of action. Because protein synthesis involves a number of time-consuming steps, intracellular receptor action usually requires a lag period of 20-30 minutes minimum and up to several hours or even days (Duval 1983). Most research indicates that the genomic effects of corticosteroids are first observed after a period of hours or days. However, some effects of glucocorticoids which are dependent on protein synthesis have been reported to occur 15-20 minutes after administration (Hallahan et al. 1973). The more rapid actions of intracellular corticosteroid receptors may work by inhibiting protein synthesis so that the time required to elicit a physiological would depend on the rate of clearance of the protein from the particular system.

Some of the best evidence that there are membrane receptors for glucocorticoids comes from work with newts (Orchinik et al. 1991). The corticosterone membrane receptor has been characterized for the roughskin newt and found to be pharmacologically distinguishable from the intracellular corticosteroid receptors (Orchinik et al. 1991). The rapid and reversible binding of corticosteroids to this receptor differs from the intracellular receptor in its affinities for specific ligands in amphibians (binding affinity for intracellular receptors in *Xenopus laevis*: dexamethasone > corticosterone: $K_d = 9.03$ nM for corticosterone [Lange & Hanke 1988] with comparable

values found for *Rana catesbeiana* [Medhi et al. 1984]; binding affinity for membrane receptors in *Taricha granulosa*: $\text{CORT} > \text{cortisol} > \text{aldosterone} \gg \text{dexamethasone}$: $K_d \sim 0.5 \text{ nM}$ [^3H]CORT [Orchinik et al. 1991]). Dexamethasone (DEX) is a synthetic glucocorticoid which acts as an agonist for both type I and type II receptors. In contrast to the type II intracellular receptor, which has high affinity for DEX in rats ($K_d = 6.2 \text{ nM}$, Anderson & Fanestil 1976) and in the clawed toad ($K_d = 3.54 \text{ nM}$, Lange & Hanke 1988), the membrane receptor for CORT in newts does not bind DEX effectively (Orchinik et al. 1991). This strong contrast between the intracellular and membrane receptor types conveniently allows experimenters to use DEX to distinguish between receptor types.

In contrast to type I and type II receptors, the current model for membrane receptors is that they elicit very rapid effects; latencies range from minutes to milliseconds (Moore & Orchinik 1991). It is hypothesized, but not proven, that many of the rapid actions of corticosteroids are mediated by membrane receptors. For example, CRF and AVP-induced ACTH and β -endorphin release, shortened duration of ethanol-induced sleep, inhibition of neuronal firing have also been evidenced to be non-genomic and presumably membrane receptor mediated actions (Jones and Hillhouse 1976, Liu et al. 1995, Liu and Chen 1995, Sze 1993, Hua and Chen 1989, Rose et al. 1995, Moore and Miller 1985, Moore and Orchinik 1994).

In most cases, actions of corticosteroids have not been directly linked to a specific membrane receptor. However, data for the rapid suppression of sexual behaviors by CORT showed a strong correlation to binding of the characterized membrane receptor

(Orchinik et al. 1991). The affinities of corticoids for [³H]CORT binding sites in synaptic membranes were shown to be linearly related to their potencies in rapid suppression of male reproductive behavior (Orchinik et al. 1991).

In many cases, corticosteroids are documented to suppress the actions of CRF. Negative feedback on the hypophysiotropic actions of CRF attenuate the stress response by preventing continued ACTH release. In fast negative feedback on CRF in mammals, it has been suggested, but not demonstrated, that glucocorticoids act on a plasma membrane to affect the conformation of the CRF receptor and affect coupling to second messengers (Childs and Unabia 1990). Intermediate feedback effects are thought to involve rapidly synthesized protein which modify intracellular calcium signals (Antoni et al. 1992). Delayed feedback is also believed to be regulated by intracellular receptor regulation of ACTH release (Childs and Unabia 1990).

Rose (1997) showed that CORT administration can suppress CRF-induced neuronal firing in newts. These data, however, showed that the direction of the effect (suppression or enhancement) of CORT on CRF-induced neuronal firing depends on the time sequence and order in which these two chemical messengers are administered. When CORT was injected 5 minutes before the CRF injection, or when it was injected 30 minutes following the CRF injection, CRF-induced increase in neuronal activity was suppressed by CORT. However, if the CORT was injected 5-10 minutes after the CRF injection, there was a potentiation in neuronal activity.

In summary, previous studies have shown that CRF can act centrally to enhance

locomotor activity in animals exposed to harsh or stressful conditions. There also is evidence that many of the CRF actions are counteracted by elevated levels of glucocorticoids and that glucocorticoids can cause rapid behavioral responses by activating the membrane receptor for corticosteroids. Therefore, using roughskin newts, the present studies ask the following questions: (i) does CORT administration prevent stress-induced increases in locomotor activity? (ii) does CORT administration prevent CRF-induced increases in locomotor activity? (iii) does the time-course and pharmacological specificity of the effects of CORT on locomotor activity fit the model for intracellular or membrane receptors?

EXPERIMENTAL METHODS

Study Animals

All experiments were performed on adult male roughskin newts (*Taricha granulosa*) which were on average 20 grams in mass and 18 cm in length. Newts were collected locally (Benton Co., OR) and held in the laboratory for three days prior to each experiment, by maintaining them in large holding tanks (85 cm in diameter) with flow-through dechlorinated water at a depth of 28 cm. There were 30 to 60 newts in each holding tank. Newts were maintained and tested in an environmentally controlled room which had a photoperiod of 12L:12D with lights on at 7:00 and an ambient water temperature of 10 °C. All experiments were performed between the hours of 10:00 and 16:00. Newts were returned to their original field site after completion of each experiment.

Injection Procedures

Intracerebroventricular injections into the third ventricle of the newt brain were given through a small hole made by a microdrill (Fine Science Tools, Inc.) through the top of the skull (Lowry 1991). The steel burr drill bit (Fine Science Tools, Inc.) was 0.9 mm in diameter which allowed for a hole large enough for the insertion of a microinjection needle (glass tubing, 1.0 mm, World Precision Instruments, Inc., pulled to a fine point). A perfusion pump (Rainin Instrument Co., Inc.) was used to regulate the volume and flow rate for injecting solutions through the micropipet. Local anesthetic

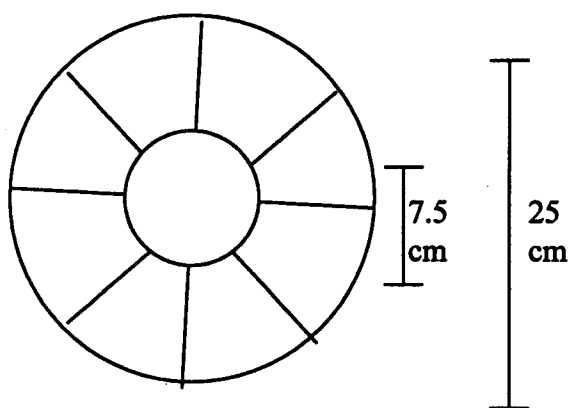
(Lanacane ®) was applied to the area of injection before and after the injection procedure. Each newt was injected approximately one minute after the other (duration of injection procedure).

Ovine corticotropin-releasing factor (Bachem Biosource, Inc.) was dissolved in Amphibian Ringers (isotonic saline). The dosage of CRF was based on prior work by Lowry (1990) which showed that 25 ng was a submaximal dose which still resulted in significant increases in locomotor activity and that a 50 ng dose yielded the maximal effect. The stock solution of corticosterone (Δ^4 -pregnene-11 β , 21-diol-3,20-dione) was prepared by dissolving CORT (Sigma Chemical Co.) in dimethyl sulfoxide (DMSO) (Fisher Chemical, Fisher Scientific) at a concentration of 20 nM. The stock solution was diluted to give a final injection concentration of 0.2 nmol CORT and 1% DMSO for Experiment 1 and 0.1 nmol CORT and 0.5% DMSO for experiments 2 and 3. Dexamethasone (9 α -fluoro-16 α -methyl prednisolone, DEX) (Sigma Chemical Co.) was prepared like CORT was, to produce a final concentration of 0.2 nmol DEX and 1% DMSO for Experiment 1 and 0.1 nmol and 0.5% for experiments 2 and 3. Vehicle injection solutions for both CORT and DEX consisted of 1% DMSO for experiment 1 and 0.5% DMSO for experiments 2 and 3 dissolved in Amphibian Ringers.

Testing Arena

All experiments used a standard testing arena to measure locomotor activity. Locomotor activity in newts consists of combined swimming and walking movements underwater. Methods and behavioral testing apparatus were similar to those described by

Lowry and Moore (1991). The testing arenas were made of opaque white plastic and had a height of 30 cm, an outside diameter of 25 cm, and an inside diameter of 7.5 cm. Each testing arena contained dechlorinated water to an approximate depth of 10 cm. Radial lines on the bottom surface of the arenas marked 8 equal sections and were used to measure locomotor activity. Final measurements were recorded as the number of line crossings per three minute interval. Each testing arena contained only one newt during experiments and data collection.



Behavioral Tests

Locomotor activity was recorded on video tape using an overhead camera to simultaneously monitor 20 testing arenas and a clock to record real time. Because only 20 arenas could be monitored at one time by the camera, experiments were divided into 2 to 4 time blocks. Each time block contained equal numbers of animals from all treatment groups (e.g. 5 animals from each of 4 treatment groups). Each experiment consisted of three to four blocks to yield sample sizes of between 15 to 20 newts per treatment group (60 to 80 newts in experiments with four treatment groups).

Newts were allowed to walk and swim around freely in their arena for one hour

while being video taped. Locomotor activity data were then collected from the video tape and recorded as the number of line crossings per 3 minute interval. For experiment 1, locomotor activity for each newt was recorded as the total number of line crossings for the three minutes immediately preceding handling stress and then for the three minutes immediately following handling stress. For experiments 2 and 3, the total number of line crossings per three minute interval was determined, starting at time zero (immediately after the injection and placement into the testing arena), for 7 or 10 consecutive 3-minute time intervals (a total of 21 or 30 minutes). Collecting the data in three minute intervals allowed for direct comparison of the locomotor activity of CRF-treated animals with values found in CRF experiments performed by Lowry (1990).

Statistical Analysis

Paired t-tests were used to analyze data from experiment 1, comparing locomotor activity during the 3 minutes before handling with the locomotor activity during the 3 minutes following handling stress. To compare the effect of the corticosteroids on spontaneous activity (pre-handling levels) and on stress-induced activity (post-handling levels minus pre-handling levels) of vehicle-treated animals to steroid-treated animals in experiment 1 unpaired t-tests were used. For experiments 2 and 3, the total number of line crossings per 3 minute interval for intervals 3 through 7 (minutes 6-21) were averaged for each animal to calculate the mean number of line crossings per three minutes for each animal. Mean values in experiments 2 and 3 were analyzed using a two-way ANOVA or a one-way ANOVA followed by Tukey HSD post-hoc test.

RESULTS AND SPECIFIC METHODS

Experiment 1

Experiment 1 was designed to investigate the effects of CORT (or DEX) administration on locomotor activity before and after the animals were exposed to a standardized handling stress. Each animal was removed individually from the holding tank, given an icv injection, and then immediately placed into its own testing arena. In experiment 1A, newts received a single icv injection of CORT or vehicle (Fig. 1A). Experiment 1A was performed on Dec 18, 1996 on newts collected on Dec 15. In experiment 1B, newts received a single icv injection of either DEX or vehicle (Fig 2A). Experiment 1B was performed on Dec 20 on newts collected Dec 17. Twenty minutes following the injection and 2 hours after the injection, each newt received a standardized handling stress for a duration of one minute. The handling-stress procedure consisted of holding the newt behind the forelimbs and atop the palm of the other hand while applying gentle pressure to the head with the thumb. This handling procedure was developed and described by Lowry and Moore (1991) and served to restrain rather than hurt the newt. Newts were subjected to the handling procedure at 20 minutes after the injection because it allowed ample time for the steroid to take effect but, most likely, not enough time for effects to result from protein synthesis. It also matched the time interval used by Orchinik et al. (1991) to investigate the rapid inhibitory effects of CORT on newt courtship behaviors. Locomotor activity for each newt was recorded for the 3 minutes

Fig. 1 The effect of CORT administration on handling stress-induced locomotor activity of male roughskin newts. Animals received either vehicle or CORT icv injection. **A.** Experimental design. **B.** Statistical analysis and significant results. **C.** Handling stress applied 20 minutes after icv administration of CORT. Locomotor activity calculated as mean number of line crossings during the 3 minutes immediately preceding handling and for the 3 minutes following handling (mean \pm SE). **D.** Difference in locomotor activity calculated as after handling minus before handling stress (mean \pm SE) at 20 minutes post-injection. **E.** Locomotor activity (mean \pm SE) in response to handling stress applied 2 hours after icv injection. **F.** Difference in locomotor activity calculated as after handling minus before handling stress (mean \pm SE) at 2 hours post-injection.

A. Experimental design *

	vehicle	69 ng CORT
handling stress at 20 min & 2 hr	n = 24	n = 24

*Injections were given in quantities of 1 μ l.

B. Statistics

Paired t-test *Activity before vs. : vehicle at 20 min*: $t = 2.3$, $df = 23$, $p < 0.03$;

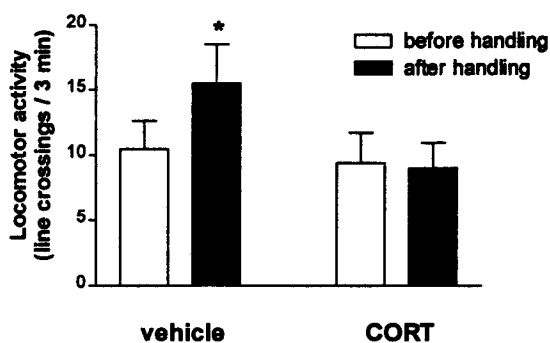
*after handling** : vehicle at 2 hr: $t = 3.6$, $df = 19$, $p < 0.002$;

: CORT at 2 hr: $t = 4.1$, $df = 14$, $p < 0.001$.

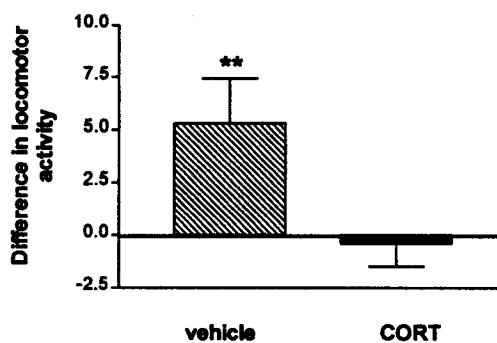
Student's t-test *Vehicle vs. CORT difference in activity*** : $t = 2.4$; $df = 22$; $p < 0.02$.

*20 min vs. 2 hr prehandling activity**** : $t = -2.2$; $df = 14$; $p < 0.05$.

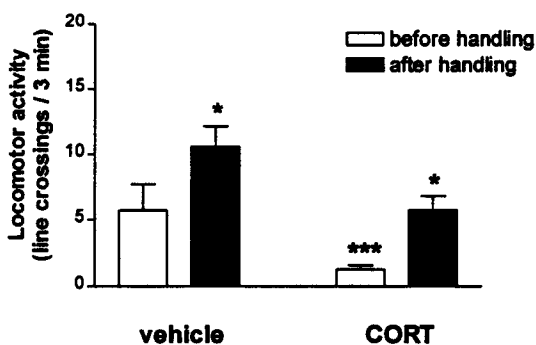
C. Stress at 20 min postinjection



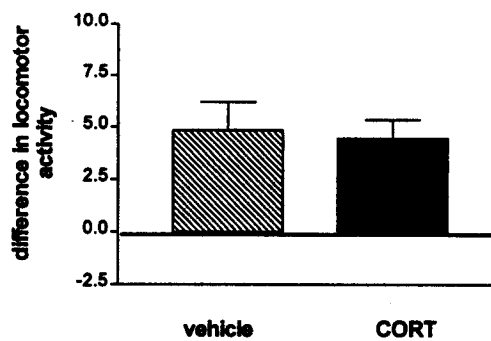
D. Stress at 20 min postinjection



E. Stress at 2 hrs postinjection



F. Stress at 2 hrs postinjection



A. Experimental design *

	vehicle	69 ng CORT
handling stress at 20 min & 2 hr	n = 24	n = 24

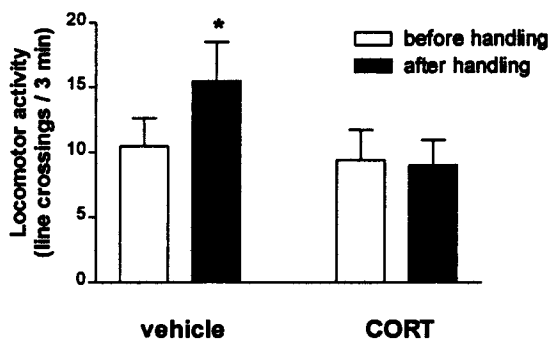
*Injections were given in quantities of 1 μ l.

B. Statistics

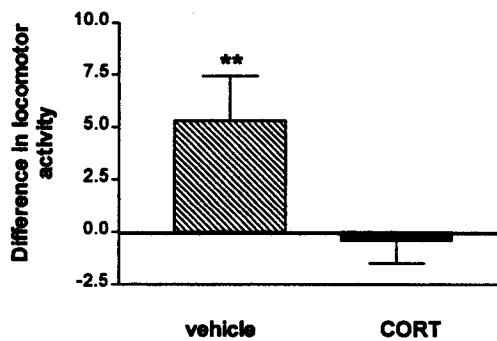
Paired t-test *Activity before vs. :* **vehicle at 20 min:** $t = 2.3$, $df = 23$, $p < 0.03$;
*after handling** : **vehicle at 2 hr:** $t = 3.6$, $df = 19$, $p < 0.002$;
 : **CORT at 2 hr:** $t = 4.1$, $df = 14$, $p < 0.001$.

Student's t-test *Vehicle vs. CORT difference in activity** :* $t = 2.4$; $df = 22$; $p < 0.02$.
*20 min vs. 2 hr prehandling activity*** :* $t = -2.2$; $df = 14$; $p < 0.05$.

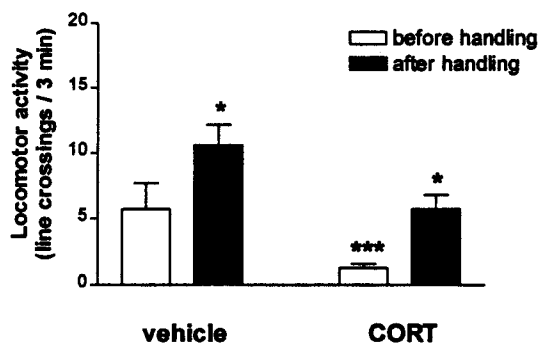
C. Stress at 20 min postinjection



D. Stress at 20 min postinjection



E. Stress at 2 hrs postinjection



F. Stress at 2 hrs postinjection

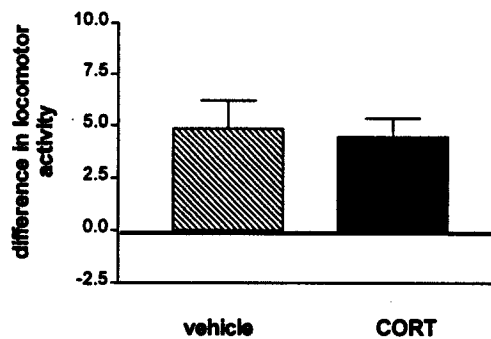


Fig. 2 The effect of DEX administration on handling stress-induced locomotor activity of male roughskin newts. Animals received either vehicle or DEX icv injection. **A.** Experimental design. **B.** Statistical analysis and significant results. **C.** Handling stress applied 20 minutes after icv administration of DEX. Locomotor activity calculated as mean number of line crossings during the 3 minutes immediately preceding handling and for the 3 minutes following handling (mean \pm SE). **D.** Difference in locomotor activity calculated as after handling minus before handling stress (mean \pm SE) at 20 minutes post-injection. **E.** Locomotor activity (mean \pm SE) in response to handling stress applied 2 hours after icv injection. **F.** Difference in locomotor activity calculated as after handling minus before handling stress (mean \pm SE) at 2 hours post-injection.

A. Experimental design ★

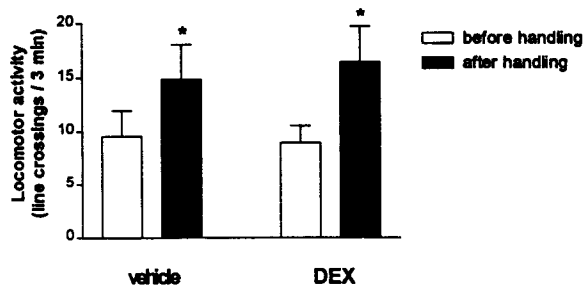
	vehicle	69 ng CORT
handling stress at 20 min & 2 hr	n = 23	n = 23

★ Injections were given in quantities of 1 μ l.

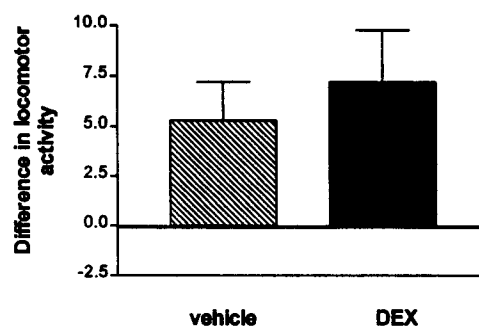
B. Statistics

Paired t-test Activity before vs. : vehicle at 20 min: $t = 2.8$; $df = 19$, $p < 0.01$;
*after handling** : DEX at 20 min: $t = 2.9$, $df = 19$, $p < 0.01$;
: vehicle at 2 hr: $t = 4.2$, $df = 19$, $p < 0.0005$;
: CORT at 2 hr: $t = 4.1$, $df = 19$, $p < 0.0007$.

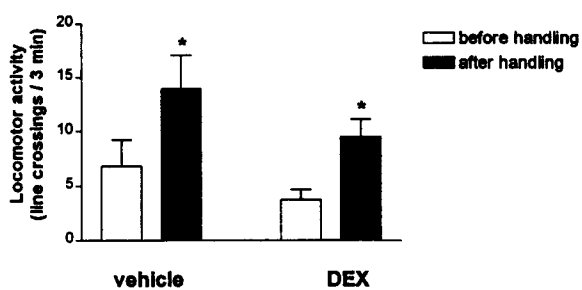
C. Stress at 20 min postinjection



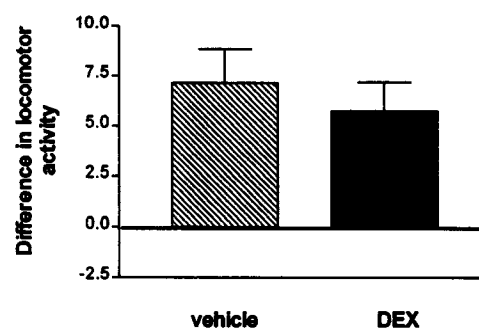
D. Stress at 20 min postinjection



E. Stress at 2 hrs postinjection



F. Stress at 2 hrs postinjection



before and the 3 minutes after the handling stress as the number of line crossings per 3-minute interval. Data were collected from a video recording for the times mentioned above.

Results are presented in Fig 1 and Fig 2. Experiment 1A revealed that a low dose of CORT, administered centrally, rapidly prevented the stress-induced increase in locomotor activity. Mean differences in locomotor activity (before and after handling) for CORT were significantly less than differences for vehicle at 20 minutes, but not at 2 hours (Fig 1D & 1F). A paired t-test comparison of the activity levels before and after handling for CORT- and vehicle-treated newts showed a significant suppression of handling-induced locomotor activity by CORT at 20 minutes but not at 2 hours (Fig 1C & 1E). Handling-induced locomotor activity was significantly higher than pre-handling values in vehicle-treated animals at both times and in CORT-treated animals at 2 hours. In addition, pre-handling activity levels were significantly lower in CORT-treated animals at 2 hours in comparison to vehicle-treated newts.

Mean differences in locomotor activity for DEX-injected newts, however, were not significantly different from the differences for vehicle-injected animals at 20 minutes or 2 hours (Fig 2D & 2F). In addition, in response to the handling procedure, DEX-treated newts showed significant increases in locomotor activity that were similar to and not statistically different from increases in vehicle-treated animals at both times tested (Fig 2C & 2E).

These results show that the central administration of CORT, but not DEX, is

capable of preventing handling-induced increases in locomotor activity as well as suppressing spontaneous locomotor activity. The decrease in spontaneous locomotor activity occurred only after 2 hours after CORT administration. The prevention of stress-induced increases in locomotor activity, however, is rapid and transient: occurring 20 minutes after the CORT injection but not at two hours after the injection.

Experiment 2

Experiment 2 was designed to investigate the effects of CORT (or DEX) administration on CRF-induced locomotor activity. Previous work indicated that the increased locomotor activity associated with the handling-stress procedure with newts involves endogenous CRF (Lowry 1991). Rose et al. (1997) also found that CORT can modify CRF-induced changes in neuronal activity. Therefore, it seemed reasonable that the results from experiment 1 indicated that CORT may prevent CRF-induced increases in locomotor activity.

As in experiment 1, each animal was removed individually from the holding tank, received one 2.0 μ l icv injection containing one of the four treatments, and was then immediately placed into its own testing arena. In experiment 2A, to evaluate CORT, CRF interaction, newts received a single icv injection of vehicle, CORT, CRF, or CRF plus CORT (Fig 3A & 4A). Experiment 2A-1 was performed on Oct 11, 1996 on newts collected on Oct 8. Experiment 2A-2 was performed on Dec 11, 1996 on newts collected on Dec 8. In experiment 2B, which was performed on Feb 7 on newts collected on Feb 4, newts received an icv injection of vehicle, DEX, CRF, or CRF plus DEX (Fig 5A) to

evaluate DEX x CRF interaction.

Fig. 3 Effects of CORT on CRF-induced locomotor activity in male roughskin newts. Vehicle, CRF, CORT or CRF plus CORT administered in a single icv injection. **A.** Experimental design. **B.** Statistical analysis and results. **C.** Time course showing number of line crossings for the 30 minutes immediately following the icv injection divided into 10 consecutive 3-minute intervals. **D.** Mean locomotor activity per 3 minute interval calculated as the mean of the average of intervals 3 -7 (minutes 6 - 21) for each newt (mean \pm SE).

A. Experimental design★

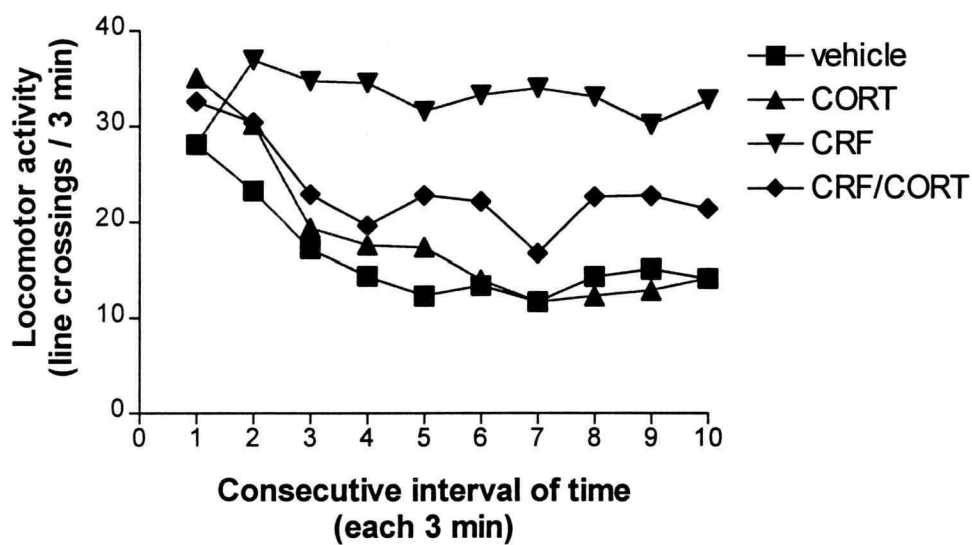
	vehicle	25 ng CRF
vehicle	n = 15	n = 15
69 ng CORT (0.2 nmol)	n = 15	n = 15

B. Statistics

2-way ANOVA	
<i>CRF main effect:</i>	
F(55,1) = 16.1; p < 0.001	
<i>CORT main effect:</i>	
F(55,1) = 2.95; p < 0.092	
<i>CRF x CORT interaction:</i>	
F(55,1) = 5.95; p < 0.018	

★Injections were given in quantities of 2 μ l.

C.



D.

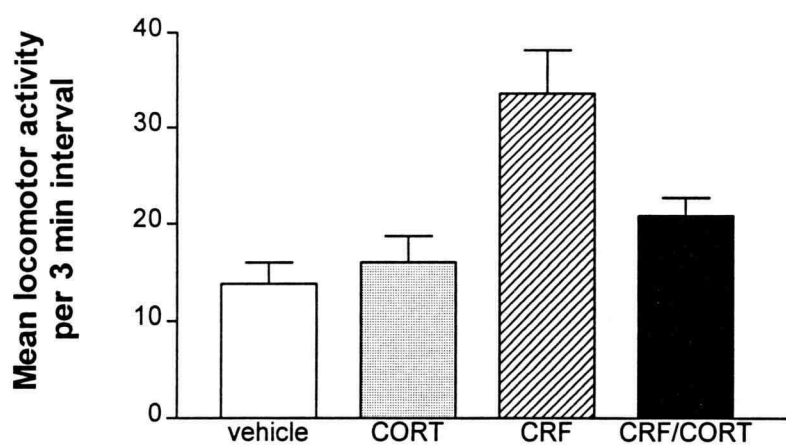


Fig. 4 Effects of CORT on CRF-induced locomotor activity in male roughskin newts. Vehicle, CRF, CORT, or CRF plus CORT administered in a single icv injection. **A.** Experimental design. **B.** Statistical analysis and results. **C.** Time course showing number of line crossings for the 21 minutes immediately following the icv injection divided into 7 consecutive 3-minute intervals. **D.** Mean locomotor activity per 3 minute interval calculated as the mean of the average of intervals 3 -7 (minutes 6 - 21) for each newt (mean \pm SE).

A. Experimental design ★

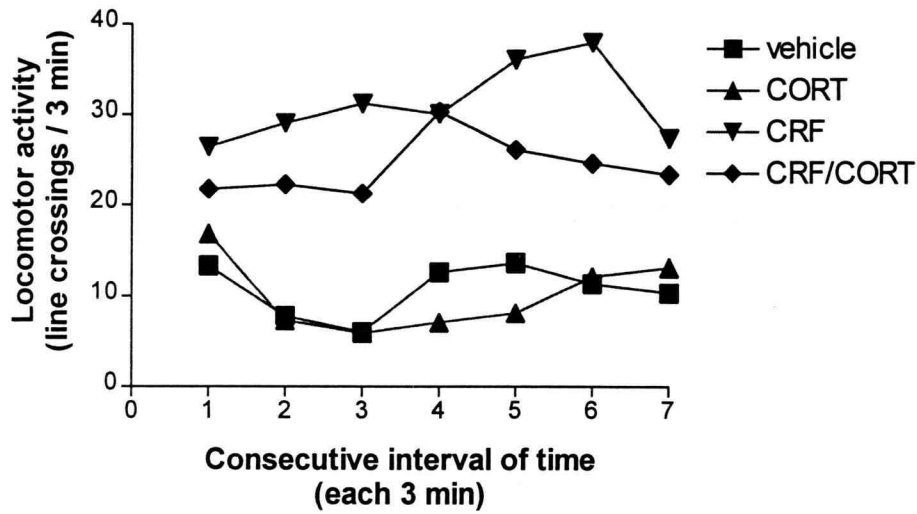
	vehicle	25 ng CRF
vehicle	n = 15	n = 15
69 ng CORT (0.2 nmol)	n = 15	n = 15

B. Statistics

2-way ANOVA	
<i>CRF main effect:</i>	
F(56,1) = 29.6; p < 0.001	
<i>CORT main effect:</i>	
F(56,1) = 1.69; p < 0.20	
<i>CRF x CORT interaction:</i>	
F(56,1) = 0.75; p < 0.39	

★ Injections were given in quantities of 2 μ l.

C.



B.

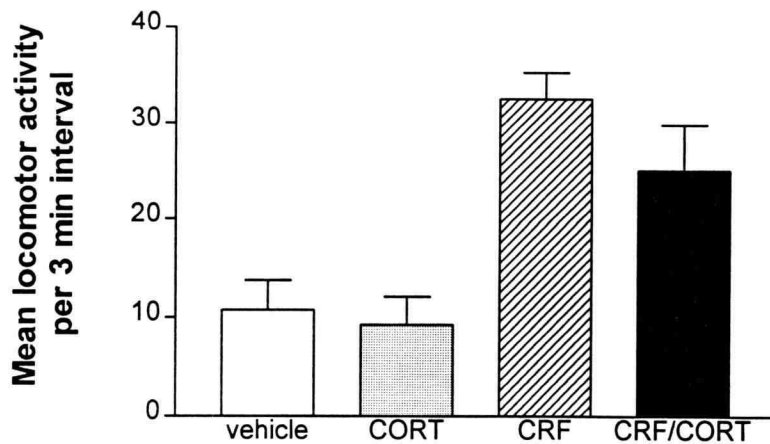


Fig. 5 Effects of DEX on CRF-induced locomotor activity in male roughskin newts. Vehicle, CRF, DEX, or CRF plus DEX administered in a single icv injection. **A.** Experimental design. **B.** Statistical analysis and results. **C.** Time course showing number of line crossings for the 30 minutes immediately following the icv injection divided into 10 consecutive 3-minute intervals. **D.** Mean locomotor activity per 3 minute interval calculated as the mean of the average of intervals 3 -7 (minutes 6 - 21) for each newt (mean \pm SE).

A. Experimental design ★

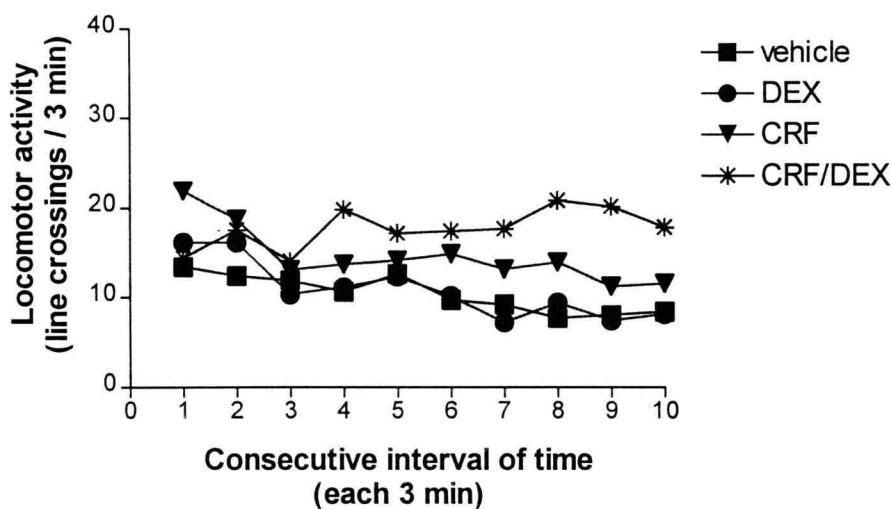
	vehicle	25 ng CRF
vehicle	n = 15	n = 15
78 ng DEX (0.2 nmol)	n = 15	n = 15

B. Statistics

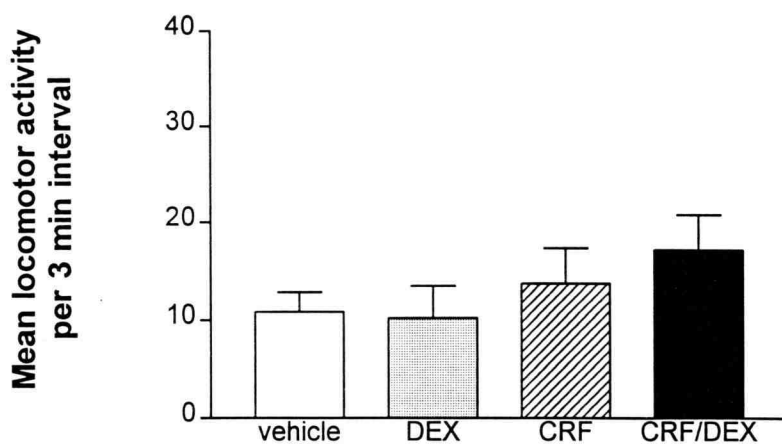
2-way ANOVA	
<i>CRF main effect:</i>	
F(56,1) = 2.54; p < 0.12	
<i>DEX main effect:</i>	
F(56,1) = 0.21; p < 0.65	
<i>CRF x DEX interaction:</i>	
F(56,1) = 0.43; p < 0.51	

★Injections were given in quantities of 2 μ l.

C.



D.



Results from experiment 2 demonstrate that CORT administration has the ability to rapidly inhibit CRF responses. The exact nature and strength of this interaction, even the presence of any interaction at all, appears to be variable however. In experiments 2A-1 and 2A-2, locomotor activity was significantly elevated in newts that received the CRF injection as indicated by significant F values for CRF main effect (Figs 3B & 4B). In experiment 2A-1, but not 2A-2, the CORT injection suppressed the CRF-induced increases in locomotor activity, as indicated by the significant F value for the CRF CORT interaction (Fig 3B).

Experiment 2B was designed to investigate the specificity of the inhibitory effects of CORT, by testing the effects of DEX, which does not bind to the CORT membrane receptor. The results of experiment 2B, however, are inconclusive, because in this experiment CRF-injected newts showed an overall smaller increase in locomotor activity than previous experiments (Fig 5) and failed to show a significant elevation in locomotor activity in response to CRF. Therefore, the inhibitory effect of DEX administration on CRF-induced locomotion could not be assessed.

Results of experiment 2 showed that CORT injection, in some cases, can significantly and rapidly suppress CRF-induced locomotor activity, but further experiments are necessary to determine if this effect is specific for CORT and not DEX.

Experiment 3

Experiment 3 was conducted on Apr 7 and 8, 1997, on newts which were collected on Apr 4 and 5, 1997. This experiment combined experiments 2A and 2B into

one large experiment. Because the results of experiment 2B and a subsequent pilot study indicated that newts might be less responsive to CRF during this time of year, experiment 3 used a higher dose (50 ng / μ l) of CRF than was used in experiment 2. Other procedures were identical to those for experiment 2, and newts received one of the following injections: vehicle, CORT, DEX, CRF plus CORT, or CRF plus DEX (Fig 6A). Experiment 3 tested the effects of CORT and DEX on CRF-induced locomotion in one large experiment.

Results from experiment 3 were similar to those of experiment 2B. Despite the higher dose of CRF, CRF injection failed to produce a significant increase in locomotor activity. Statistical analysis by a one-way ANOVA showed a significant overall effect, but the Tukey HSD post hoc test of the average revealed only one significant difference: a significant difference between DEX and CRF/DEX (Fig 6).

Fig. 6 Effects of CORT or DEX on CRF-induced locomotor activity in male roughskin newts. Vehicle, CRF, CORT, CRF plus CORT, DEX, or CRF plus DEX administered in a single icv injection. **A.** Experimental design. **B.** Statistical analysis and significant results. **C.** Time course showing number of line crossings for the 30 minutes immediately following the icv injection divided into 10 consecutive 3-minute intervals. **D.** Mean locomotor activity per 3 minute interval calculated as the mean of the average of intervals 3 -7 (minutes 6 - 21) for each newt (mean \pm SE).

A. Experimental design ★

	vehicle	25 ng CRF
vehicle	n = 20	n = 20
69 ng CORT (0.2 nmol)	n = 20	n = 20
78 ng DEX (0.2 nmol)	n = 20	n = 20

B. Statistics

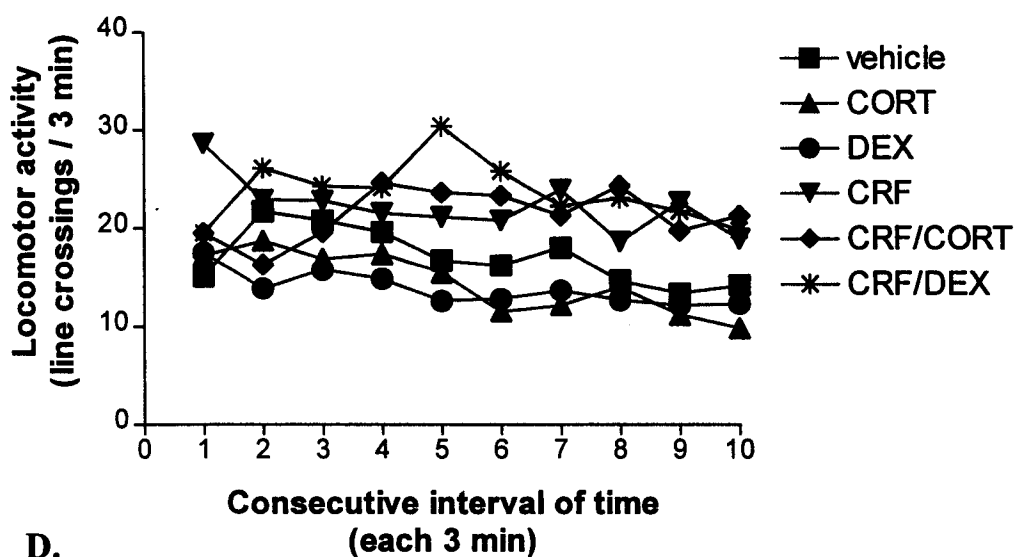
One-way ANOVA

$F(110,5) = 3.03; p < 0.013$

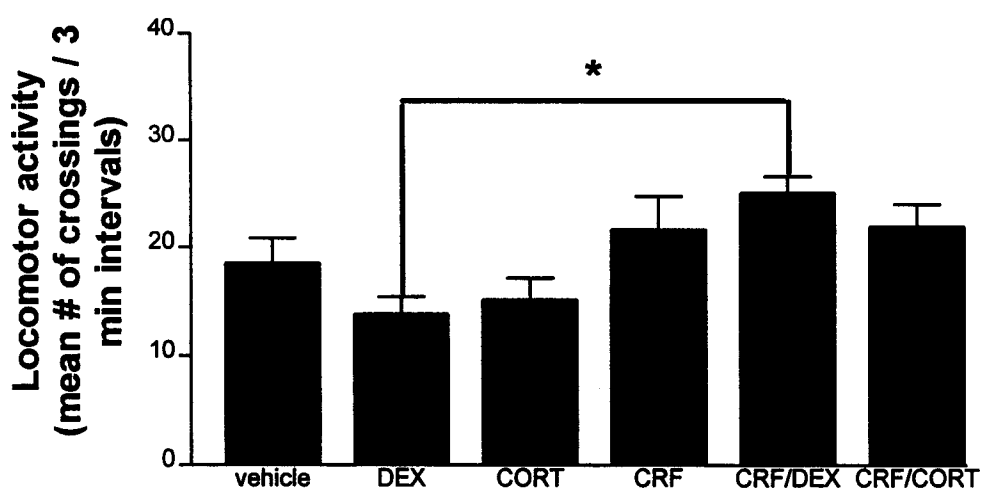
Tukey HSD post hoc test
yielded one significant
difference (*): $p < 0.034$

★ Injections were given in quantities of 2 μ l.

C.



D.



DISCUSSION

In experiment 1, exposure of control newts to the handling-stress procedure increased post-handling locomotor activity levels by approximately 83% above the pre-handling levels of locomotor activity. In previous studies (Lowry and Moore 1991), the same handling-stress procedures produced an increase in locomotor activity in vehicle-injected newts of approximately 180 % between pre- and post-handling levels. Thus, in both studies the handling-stress resulted in significant increases in locomotor activity in roughskin newts, even though the magnitude of the responses were not the same.

Results from experiment 1 demonstrated that CORT, but not DEX, can rapidly prevent the effects of handling-stress on locomotor activity. At 20 minutes, the handling-induced increase in locomotor activity was prevented by CORT. Because DEX failed to cause the same response as CORT, and because CORT but not DEX binds to the membrane receptor (Orchinik et al. 1991), these results indicate that the rapid prevention of stress-induced increases in locomotor activity is specific, not common to all glucocorticoids, and may involve the membrane receptor.

At 2 hours after the steroid injection in experiment 1, CORT no longer affected stress-induced increases in locomotor activity, demonstrating that this rapid effect of CORT is also transient, lasting less than 2 hours. CORT did, however, cause a decrease in spontaneous locomotor activity from that of vehicle-treated newts. DEX did not influence the effect of handling on locomotor activity 2 hours after the injection, nor did

it significantly lower spontaneous locomotor activity. The absence of any effect of DEX on stress-induced increases in locomotor activity is consistent with the notion that intracellular type II receptors are not involved in these behavioral responses. Type II receptors have been shown to bind DEX with higher affinity than they bind CORT (Reul and de Kloet 1985, Orchinik et al. 1991).

The temporary nature of CORT's effect in experiment 1 is probably caused by the small dose, which was most likely quickly diluted and cleared from the newt's system, causing only a short-term activation of the receptor mechanisms. The dose of CORT that was injected (icv) was small (0.2 nmol = 69 ng): 20 times smaller than the ED_{50} (4 nmol) of CORT given ip for inhibiting clasping behavior in newts (Orchinik et al. 1991). Therefore, prevention of handling-induced increases in locomotor activity by CORT is rapid, specific, and transient. In addition, since rapid recovery of the system following steroid removal is considered characteristic of a non-genomic effect (Duval et al. 1983), these results suggest that this prevention by CORT is through non-genomic mechanisms.

The rapidity and specificity of this CORT effect on locomotor activity suggests that this response is mediated through the membrane receptor that was characterized by Orchinik et al. (1991) in the roughskin newt. There is evidence that CORT membrane receptors exist in other vertebrate taxa. Towle and Sze (1983) demonstrated that there are specific binding sites for CORT in synaptic plasma membranes in rat brains. Hua and Chen (1989) provided further evidence for the existence of a membrane receptor by studying physiological responses in guinea pig ganglion neurons. In addition, the rapid

inhibition of prolactin release by cortisol (a major corticosteroid in teleost fishes) in male Tilapia, has been suggested to work by “reducing the influx of extracellular Ca^{2+} through plasma membrane-associated Ca^{2+} channels” (Borski et al. 1991).

Most evidence for membrane receptors comes from previous studies that document the rapidity of some of the actions of corticosteroids. Administration of various glucocorticoids has been shown to inhibit CRF-induced ACTH release (Antoni et al. 1992, Britton et al. 1986, Childs and Unabia, 1990, Dallman and Yates 1969, Hallahan et al. 1989, Hinz and Hirschelmann 1995, Jones et al. 1972, Widmaier and Dallman 1984). Administration of glucocorticoids has also been demonstrated to affect neuronal firing within seconds to minutes (Rose et al. 1995, French-Mullen 1995, Orchinik et al. 1994). The suppression of courtship behaviors by CORT in male roughskin newts was shown to occur within 8 minutes of an ip CORT injection (Orchinik et al. 1991). Neuronal firing in response to courtship behavioral stimuli was suppressed 10-15 minutes after CORT administration (Orchinik et al. 1994). In neurophysiological studies in newts, suppression of AVT- and CRF-stimulated medullary firing by CORT occurred within 1-5 minutes after ip administration of CORT (Rose et al. 1995). The rapidity of this response and others suggests that the interactions between CORT and CRF may involve non-genomic actions.

There are differences in binding affinity and pharmacological specificity between the intracellular and membrane receptors for corticosteroids. Intracellular receptors bind both CORT and DEX with high affinity, and the type II receptor binds DEX with high

affinity (Reul and de Kloet 1985, Orchinik et al. 1991). In contrast, the CORT membrane receptor in newts binds DEX with extremely low affinity (Orchinik et al. 1991). This distinct difference in pharmacological signature between the intracellular and membrane receptors provides an experimental approach to distinguish between these two types of receptors. The work of Orchinik et al. (1991) provides an example where a specific behavioral response is associated with the membrane receptor for CORT. That study found a strong correlation between the binding affinities of various glucocorticoids (including CORT and DEX) to the CORT membrane receptor and the potency of these glucocorticoids to inhibit sex behaviors in newts within 20 minutes of the injection. The results from experiment 1, showing that CORT and not DEX suppresses handling-induced locomotor activity at 20 minutes and that this effect no longer exists at 2 hours post-injection, is consistent with the hypothesis that these inhibitory effects of CORT are mediated by the membrane receptor.

There is evidence that other systems, besides newt behaviors, may involve non-genomic effects of CORT. Experiments on rat hypothalamic slices *in vitro* showed that corticosteroid administration can inhibit AVP release within 20 minutes in a dose dependent manner. This rapid effect of CORT is not affected by drugs that inhibit gene transcription, protein synthesis, or axoplasmic transport, indicating that this rapid effect is non-genomic (Liu et al. 1995, Liu and Chen 1995). Experiments by Sze (1993) showed that corticosteroid administered to rats 15 minutes before administration of a sleep-inducing dose of ethanol significantly shortened the sleep time in a dose-dependent

manner. Blood ethanol levels were the same across all treatments, demonstrating that the corticosteroids were not acting through a change in the metabolism of ethanol. Also, corticosterone administered in a dose of 20 mg / kg reduced sleeping time from 33 minutes to 14 minutes, showing that this is a rapid effect and so was interpreted as evidence for non-genomic mechanisms. Sandi et al. (1996) showed that corticosterone, at doses that mimic physiological concentrations of corticosterone in the plasma during periods of stress, induced an increase in locomotor activity in rats exposed to a novel environment; this response occurred within 7.5 minutes of the injection and was not suppressed by protein synthesis inhibitor nor by type I and type II receptor antagonists RU28318 and RU38486.

Therefore, the CORT membrane receptor seems to be involved in many rapid physiological and behavioral effects in all vertebrates, based on studies in fish, amphibians, and mammals. Rapid behavioral effects have been demonstrated in *Taricha* as well. Arginine vasotocin, a neuropeptide which has been demonstrated to enhance sexual behaviors, can be rapidly inhibited by CORT as evidenced neurophysiologically by a decrease in neuronal firing in response to sexual stimuli (Rose 1995).

Experiments 2 and 3 were conducted because it seemed likely that the action of CORT in the suppression of handling-induced locomotor behavior involved an interaction between CORT and CRF. In studies which showed that handling increases locomotor activity, Lowry (1991) showed that this increase in locomotor activity can be suppressed by an injection of the CRF antagonist, supporting the conclusion that

endogenous CRF is responsible for the increase in locomotor activity when newts are exposed to the handling-stress procedure. Consistent with this conclusion, injection of CRF antagonist in mice reversed restraint-induced suppression of exploratory behavior (Berridge and Dunn 1987), providing another example of a CRF-mediated response to restraint stress. The inhibitory actions of CORT in experiment 1 suggested that CORT might be blocking either the release of CRF or its actions.

Corticosteroids have been shown to rapidly modulate the effects of CRF in previous experiments in many species. The first report of a rapid effect of CORT was (Sayers and Sayers in 1947) was concerned with the negative feedback effects of CORT on CRF-stimulated ACTH release. The rapid phase of the negative feedback effects of glucocorticoids on ACTH secretion occurs within minutes of the administration of exogenous corticosteroids (Dallman and Yates 1969). In fact, rapid feedback has been demonstrated to occur within seconds to minutes *in vivo* and as early as within 10 minutes *in vitro* (Widmaier and Dallman 1984). In newts, corticosterone administration can suppress neuronal firing within minutes of the CRF injection (Rose 1997). Thus, CORT has been shown to interact rapidly with CRF actions in different systems, and the effects of CORT on CRF actions are typically suppressive, but not always.

Considering the above information, experiment 2 was designed to determine if CORT (or DEX) would rapidly modulate behavioral responses to exogenous CRF. Experiments 2A-1 and 2A-2 both revealed a significant effect of the CRF injection on locomotor activity (Figs 3 & 4). Mean locomotor activity was significantly higher in

CRF-injected animals than in vehicle-injected animals. These results corresponded with the increase in locomotor activity found in previous studies in newts (Moore et al. 1984; Lowry et al. 1990; Lowry and Moore 1991). Similarly, CRF administration has been found to enhance locomotor activity in rats (Sutton et al. 1982; Veldhuis and De Wied, 1984; Diamant and De Wied, 1991; Saunders and Thornhill, 1986; Sherman and Kalin, 1987).

Experiments 2A-1 and 2A-2 were intended to be replicate experiments testing the effect of CORT on CRF. CORT significantly suppressed the locomotor activity of the CRF-injected newts in experiment 2A-1 (Fig 3) but not experiment 2A-2. These results indicate that the ability of CORT to significantly suppress CRF-induced locomotor activity is variable. The difference in response could depend on unidentified differences in procedures, previous stress levels of the newts, or basal CORT levels. The reason for the inconsistent results in these experiments is unclear.

It seems that the neuroendocrine mechanism controlling stress responses are extremely complex. There are large variations in individual newts' magnitude of behavioral responses to CRF injections, handling, control injections, and containment procedures (personal observations of Lowry and Chiavarini). Rose (1997) also found variation in the magnitude and the direction of the effect of CORT administration on CRF-induced medullary firings, depending on the sequence of the injections (which came first, CORT or CRF) and exact times at which the two chemical messengers were administered. Rose (1997) observed a potentiation of the CRF-induced firing when

CORT was administered ip 5-10 minutes prior to CRF application, but a suppression of the response when CORT was administered even earlier than this or after CRF application. So, the inconsistency between experiments 2A-1 and 2A-2 may be a result of unidentified differences in the prior physiological state of the newts.

Experiment 3 was designed to further investigate the effects of CORT on CRF. However, in experiment 3 CRF injection failed to produce a significant stimulation of locomotor activity (Fig 6), in contrast to experiments 2A-1 and 2A-2. Therefore, the results of experiment 3 did not provide any additional information about the interaction between CORT and CRF.

Experiments 2B and 3 also were designed to investigate the effects of DEX on CRF-induced locomotor activity. Because the CRF injection in both of these experiments failed to cause significant behavioral effects, no conclusions could be drawn in terms of the effects of DEX on CRF-induced locomotion. Because of the lack of CRF effect, it remained undetermined whether DEX can rapidly modulate behavioral responses to CRF.

Statistical analysis of experiment 3 indicated a significant difference between the CRF/DEX and the DEX treatment groups. This appeared to be a function of both a decrease in locomotor activity in the DEX treated animals and a potentiation of the CRF-induced locomotor activity in the CRF/DEX group. The physiological significance and repeatability of this observation is unclear. If this is a repeatable effect, further studies will be required to determine the mechanisms behind these actions of DEX.

One possible reason for the failure of CRF to elicit its effects was failure of physiologically active CRF to reach its site of action. Experiments 1 and 2 were performed with aliquots from the same stock solution, stored at -80°C . Following the failure of CRF to enhance locomotor activity in experiment 2B, new CRF solution was made and two pilot experiments were performed to test the effects of CRF on locomotor activity in comparison to the effect of vehicle. These pilot experiments, like experiment 2B, failed to show the effect of CRF on locomotor activity.

The CRF dose which elicited the maximal response in dose-dependent studies by Lowry et al. (1990) was reported as 25 ng. However, balance calibration following these experiments revealed that the dose reported as 25 ng was actually 50 ng; thus, the dose of CRF which elicited a maximal response was in fact 50 ng (Lowry, personal communication). So, new CRF solution was made with a dose of 50 ng in 1 μl to determine if the dose which Lowry (1990) found to elicit the maximal CRF effect would cause significant increases in locomotor activity. Experiment 3 was performed with the 50 ng dose solution and also failed to elicit a significant increase in locomotor activity. No other indicators of the biological activity could be tested to determine the physiological activity of the CRF at this time; but, to the best of our knowledge the lack of increase in locomotor activity in response to CRF was not a result of problems with the CRF.

The experiments in which CRF failed to cause significant increases in locomotor activity were run in February and March, months which coincide with the newt breeding

season. One possible reason for differences in the effects of CRF is that breeding males respond differently to the stress of capture and being kept in the laboratory in comparison to non-breeding males. In fact, previous studies of newts support this interpretation. Deviche et al. (1990) showed that when plasma CORT concentrations were determined for newts sampled in the field, the CORT levels of male newts did not change seasonally. Moore and Zoeller (1985), however, found that plasma CORT concentrations which were measured in newts that had been held in the laboratory for one day did change seasonally, with the highest plasma CORT concentrations occurring in males during the breeding season. These data suggest that male *Taricha* are more sensitive to the stress of capture during the breeding season than out of the breeding season. Therefore, all males in experiments 2B and 3 may have had elevated plasma CORT concentrations, due to the stress of capture, prior to any experimental treatments.

So, there are several possible reasons for the failure of CRF to elicit the expected increase in locomotor activity. Technical problems could have caused these differences, though to the best of my knowledge this was not the case. Higher levels of CORT, of animals held in the laboratory during the breeding season, could prevent the CRF-induced increase in locomotor activity, as indicated by experiment 2A-1. In addition, endogenous opiates have been shown to prevent CRF-induced increases in locomotor activity and may be present in higher levels during the breeding season (Lowry et al. 1990).

Results from these three experiments show that CORT, but not DEX, can rapidly suppress handling-induced locomotor activity. The rapidity, transiency, and specificity of

this CORT effect suggests that this response is mediated through the membrane receptor that was characterized by Orchinik et al. (1991) in the roughskin newt. Results from these studies also indicate that CORT has the potential to rapidly suppress CRF-induced locomotor activity; this suppression is variable, however. In addition, the locomotor response to CRF appears to vary with the season. Because both experiments which involved DEX (experiment 2B & 3) failed to show significant increases in locomotor activity in response to CRF, no clear evidence in regards to possible rapid effects of DEX on CRF-induced locomotor activity could be demonstrated. Further studies are needed to verify the seasonal differences in response to stress, to clarify the reason for these differences, and also to clarify the role of DEX in rapid control of CRF-induced locomotor activity.

These experiments demonstrate the extreme complexity of the stress response. Differences in the stress response probably depend on the physiological condition of the animal, its prior experience, and any number of other pre-existing factors. This makes sense since newts, like other animals, must respond to a number of different stressors: predators, sudden and severe changes in weather as well as changes in environmental conditions. So, in response to stress, a newt may need to change locations, stop moving, hide, or change other behaviors or aspects of their physiology. Therefore, the ability of corticosterone to rapidly effect stress-related behaviors is a small, but important part of a larger, more complex system.

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APPENDICES

Appendix A

Background

Neurophysiological data with roughskin newts show that the direction of the effect of CORT on CRF-induced neuronal firing depends on the time sequence and order in which CORT and CRF are administered (Rose 1997). Studies by Rose demonstrated that when CORT was given before CRF and when it was given 30 minutes following CRF administration, CRF-induced neuronal firing was suppressed. If the ip injection of CORT was administered 5 to 10 minutes after the CRF application, however, neuronal firing was potentiated (Rose 1997).

Experiments A.1a and A.1b

Methods and Rationale

This experiment was run in order to test if CORT given by ip injection five minutes after an icv injection of CRF icv would potentiate CRF-induced locomotor activity, as predicted by Rose's data described above. Newts first received an icv injection of 25.0 ng CRF or vehicle in a 1.0 μ l injection. Five minutes later, each newt received an ip injection of either 25.0 μ g CORT or vehicle in a 0.1 ml injection. Vehicle for both CRF and CORT was Amphibian Ringers. Experiment A.1a was performed on July 9, 1996 on animals collected on July 6. Experiment A.1b was performed on July 31, 1996 on animals collected on July 28. Locomotor activity was recorded as line crossings per three minute interval for 20 consecutive intervals for experiment A.1a and for 7

consecutive intervals for experiment A.1b following the ip injection (see Methods pp. 10-13 for general methods).

Results and Discussion

Although no significant interaction was observed, the trend seemed to be for CORT to potentiate CRF-induced locomotor effects (Fig A.1a & A.1b), consistent with the trend in neuronal firing found by Rose (1997). The locomotor activity induced by CRF, however, was lower than in experiments in which there was no ip injection (see Fig 3 & 4 in the main body of text). It seems, then, that the ip injection confounds the data by adding an extra stressor.

Fig. A.1a Effects of CORT (ip) on CRF-induced locomotor activity in male roughskin newts. CORT given 5 minutes following CRF icv administration (replicate #1). **A.** Experimental design. **B.** Statistical analysis and results. **C.** Time course showing number of line crossings for the 2 hours immediately following the icv injection divided into 20 consecutive 3-minute intervals. **D.** Mean locomotor activity per 3 minute interval calculated as the mean of the average of intervals 3 -7 (minutes 6 - 21) for each newt (mean \pm SE).

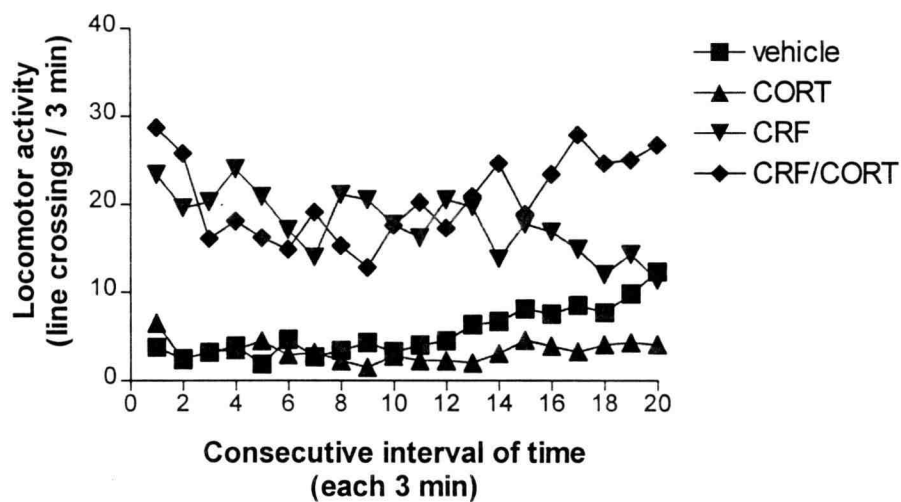
A. Experimental design

		<u>icv</u>	
		vehicle	25 ng CRF
<u>ip</u>	vehicle	n = 15	n = 15
	25 μ g CORT	n = 15	n = 15

B. Statistics

2-way ANOVA	
<i>CRF main effect:</i>	
F(1, 55) = 26.9; p < 0.001	
<i>CORT main effect:</i>	
F(1, 55) = 0.052; p < 0.82	
<i>CRF x CORT interaction:</i>	
F(1, 55) = 0.05; p < 0.82	

C.



D.

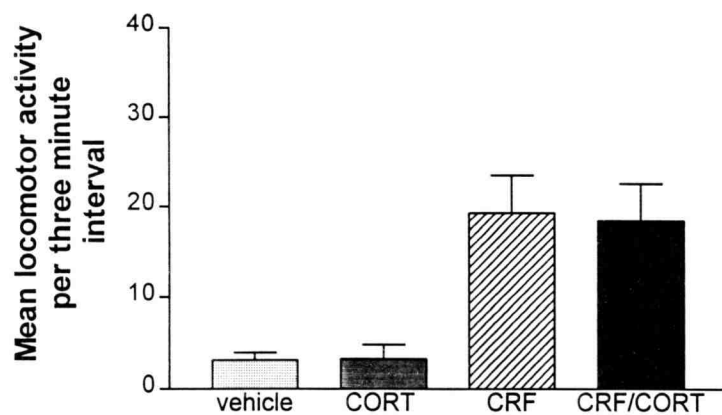


Fig. A.1b Effects of CORT (ip) on CRF-induced locomotor activity in male roughskin newts. CORT given 5 minutes following CRF icv administration (replicate #2). **A.** Experimental design. **B.** Statistical analysis and results. **C.** Time course showing number of line crossings for the 21 minutes immediately following the icv injection divided into 7 consecutive 3-minute intervals. **D.** Mean locomotor activity per 3 minute interval calculated as the mean of the average of intervals 3 -7 (minutes 6 - 21) for each newt (mean \pm SE).

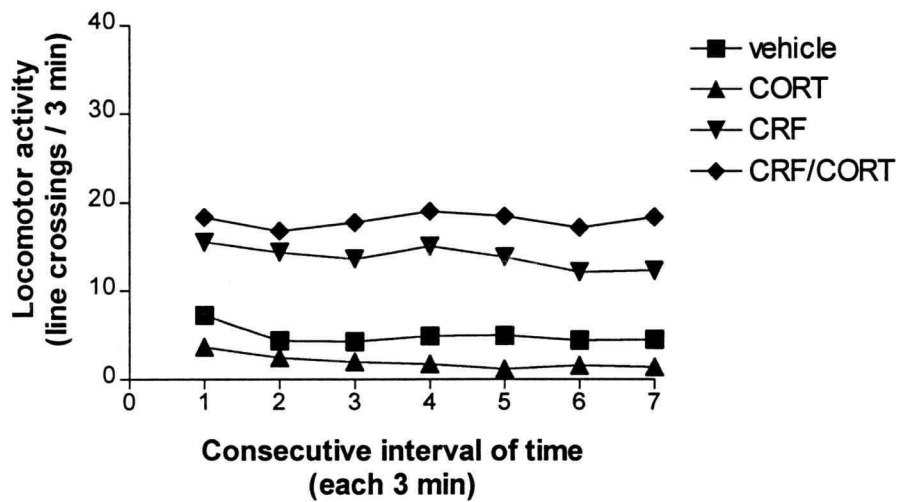
A. Experimental design

		<i>icv</i>	
		vehicle	25 ng CRF
<i>ip</i>	vehicle	n = 20	n = 20
	25 μ g CORT	n = 20	n = 20

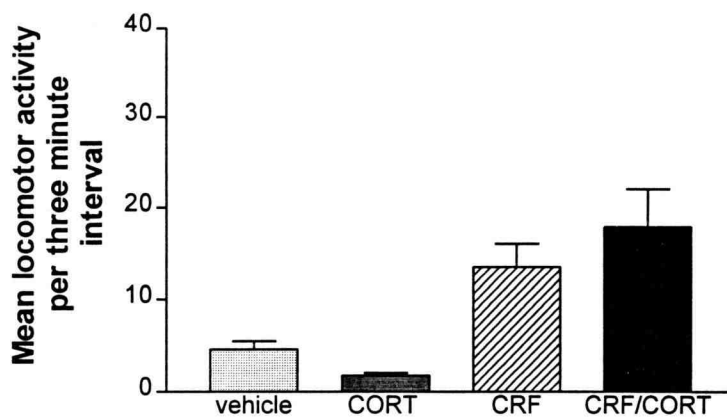
B. Statistics

2-way ANOVA	
<i>CRF main effect:</i>	
$F(1, 76) = 27.2; p < 0.001$	
<i>CORT main effect:</i>	
$F(1, 76) = 0.065; p < 0.80$	
<i>CRF x CORT interaction:</i>	
$F(1, 76) = 2.24; p < 0.14$	

C.



D.



Experiment A.2

Methods and Rationale

Experiment A.2 also aimed to test if behavioral effects of CORT ip and CRF icv administration would be consistent with CORT suppression of CRF-neuronal firing. This time, an ip injection of CORT was given 5 minutes following CRF administration. Newts first received an ip injection of either 25.0 µg CORT or vehicle in a 0.1 ml injection. Five minutes later, each newt received an icv injection of 25.0 ng CRF or vehicle in a 1.0 µl injection. Vehicle for both CRF and CORT was Amphibian Ringers. Experiment A1.2 was performed on September 23, 1996 on animals collected three days before on Sept 20. Locomotor activity was recorded as line crossings per three minute interval for 7 consecutive intervals following the ip injection (see Methods pp. 12-15 for general methods).

Results and Discussion

Again, the results were not significant. However, in contrast to neurophysiological data, CORT injection tended to affect CRF-induced responses in the direction of potentiation, rather than suppression of CRF-induced locomotor activity (Fig A.2). These results were also confounded by low locomotor activity levels in CRF-treated newts. The line of study for experiments A.1 and A.2 was abandoned because of the inability to control for the confounding effects of the double injection procedure.

Fig. A.2 Effects of CORT (ip) on CRF-induced locomotor activity in male roughskin newts. CORT given 5 minutes prior to CRF icv administration. **A.** Experimental design. **B.** Statistical analysis and results. **C.** Time course showing number of line crossings for the 21 minutes immediately following the icv injection divided into 7 consecutive 3-minute intervals. **D.** Mean locomotor activity per 3 minute interval calculated as the mean of the average of intervals 3 -7 (minutes 6 - 21) for each newt (mean \pm SE).

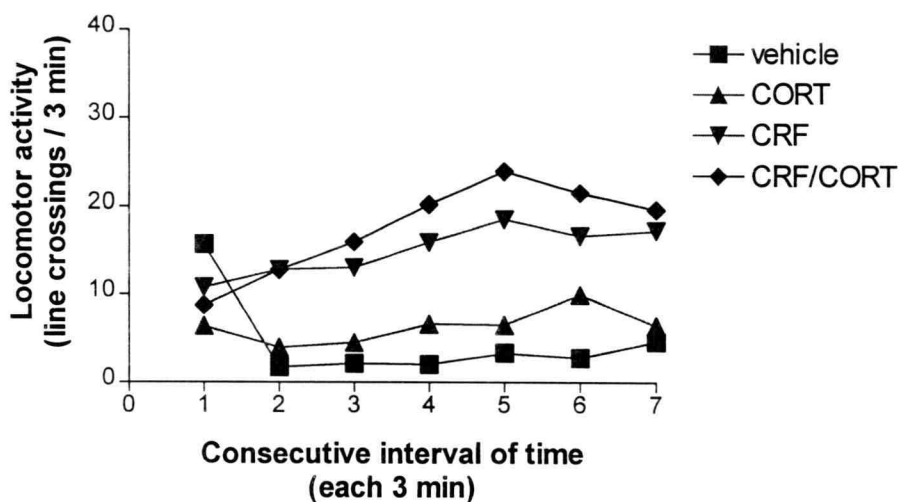
A. Experimental design

		<u>icv</u>	
		vehicle	25 ng CRF
ip	vehicle	n = 19	n = 19
	25 µg CORT	n = 19	n = 19

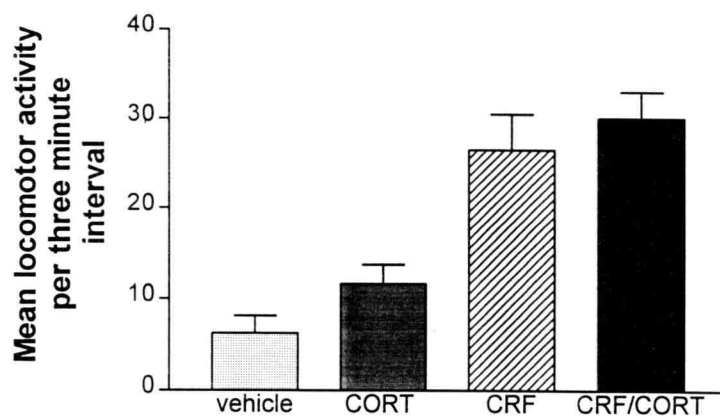
B. Statistics

2-way ANOVA	
<i>CRF main effect:</i>	
$F(1, 72) = 1.06; p < 0.31$	
<i>CORT main effect:</i>	
$F(1, 72) = 39.5; p < 0.001$	
<i>CRF x CORT interaction:</i>	
$F(1, 72) = 0.023; p < 0.88$	

C.



D.



Appendix B

Background

Serotonin concentrations have been shown to increase in the dorsomedial hypothalamus in response to either CRF or CORT administration in newts (Lowry 1995). The serotonin uptake inhibitor fluoxetine has been shown to greatly enhance CRF-induced locomotor activity (Lowry et al. 1993). It was thought that the effect of CORT on serotonin might be reflected in pronounced changes in locomotor activity when newts receive combined treatment with fluoxetine

Experiments B.1, B.2, and B.3

Methods and Rationale

CRF causes serotonin concentrations to increase in the dorsomedial hypothalamus and serotonin potentiates CRF-induced locomotor activity (Lowry 1995). Possible CORT and serotonin interaction effects on locomotor activity had not yet been investigated. Therefore, these experiments investigated the effects of CORT and fluoxetine on locomotor activity in roughskin newts. In experiment B.1, newts received either 100 ng fluoxetine or vehicle in a 1.0 μ l icv injection (a pilot study group received 100 ng of anandamide, a cannabinoid, in a 2 μ l icv injection). Five minutes later, each newt received an ip injection of either 25.0 μ g CORT or vehicle in a 0.1 ml injection. Vehicle for both fluoxetine and CORT (as well as for anandamide) was Amphibian Ringers. This experiment was performed on Aug 12, 1996 on animals collected 3 days

before on Aug 9.

Experiment B.2 and B.3 were performed as dose-response experiment. In both experiment B.2 and in experiment B.3 all newts received a 1.0 μ l icv injection of 100 ng fluoxetine. Five minutes later, each newt received an ip injection of either 0.0 μ g, 3.1 μ g, 6.25 μ g, 12.5 μ g, or 25.0 μ g CORT in 0.1 ml Amphibians Ringers. This experiment was run on Aug 29, 1996 on newts collected three days before on Aug 26. In experiment A2.3 each newt received an ip injection of either 0.0 μ g, 25.0 μ g, 50.0 μ g, 100.0 μ g, or 200.0 μ g CORT in 0.1 ml Amphibians Ringers five minutes after the icv of fluoxetine. This experiment was run on Sept 9, 1996 on newts collected three days before on Sept 6. Locomotor activity was recorded as line crossings per three minute interval for 7 consecutive intervals following the ip injection (see Methods pp. 12-15 for general methods).

Results and Discussion

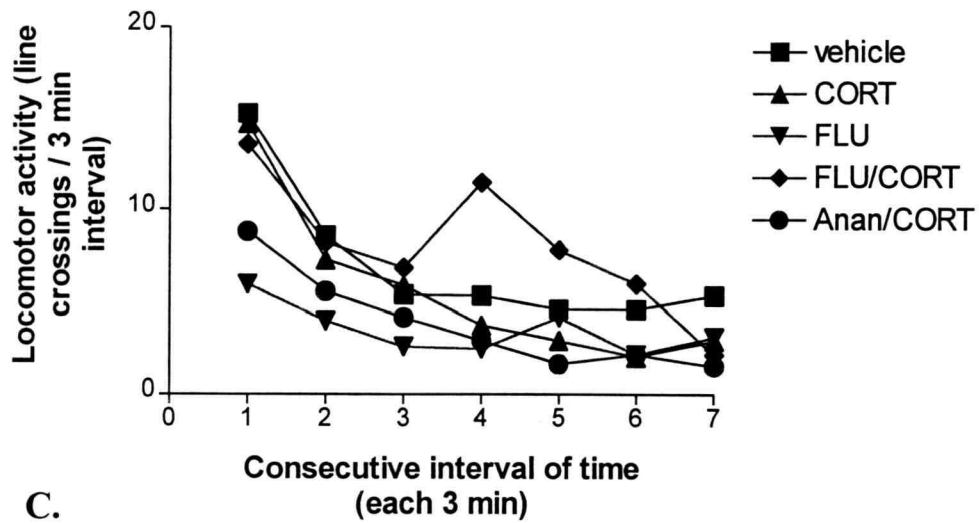
All three fluoxetine experiments failed to show any significant effect of fluoxetine or CORT administration on locomotor activity (Fig A2.1, A2.2, & A2.3).

Fig. B.1 Effects of Fluoxetine (or Anandamide) (icv) and CORT (ip) on locomotor activity in male roughskin newts. **A.** Statistical analysis and results. **B.** Time course showing number of line crossings for the 21 minutes immediately following the icv injection divided into 7 consecutive 3-minute intervals. **C.** Mean locomotor activity per 3 minute interval calculated as the mean of the average of intervals 3 -7 (minutes 6 - 21) for each newt (mean \pm SE).

A. Statistics

One-way ANOVA
 $F(4, 75) = 2.29; p < 0.067$

B.



C.

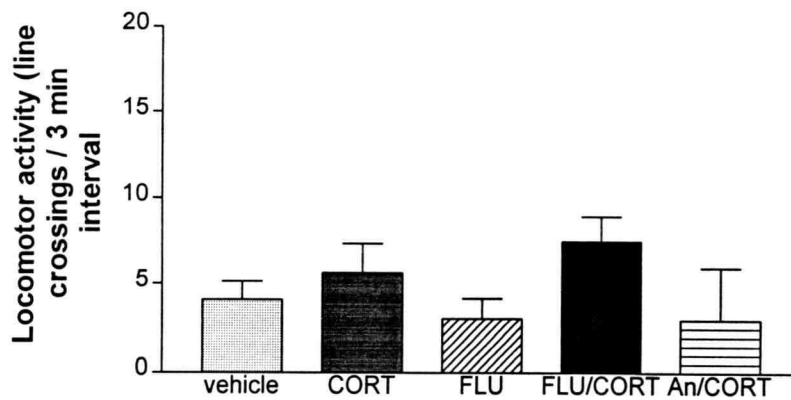


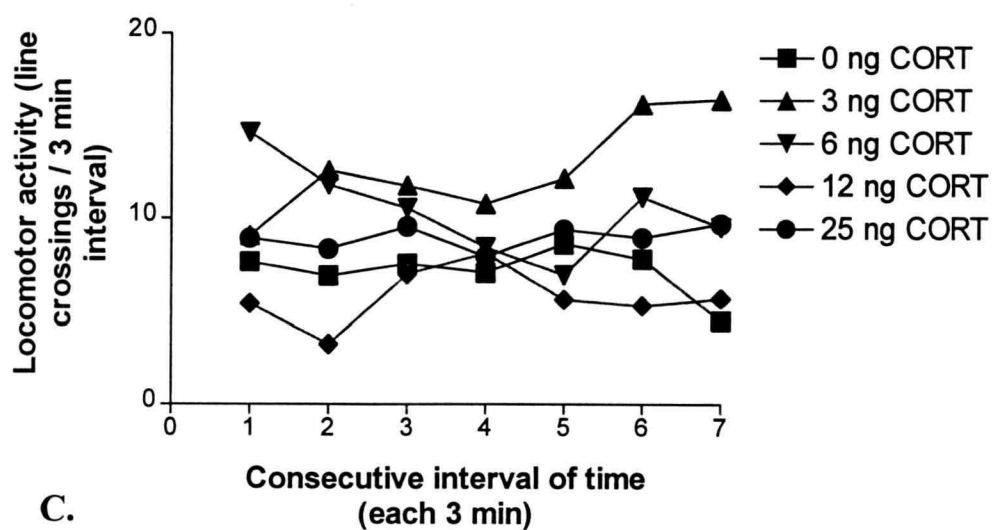
Fig. B.2 Effects of Fluoxetine (icv) and CORT (ip) on locomotor activity in male roughskin newts. **A.** Statistical analysis and results. **B.** Time course showing number of line crossings for the 21 minutes immediately following the icv injection divided into 7 consecutive 3-minute intervals. **C.** Mean locomotor activity per 3 minute interval calculated as the mean of the average of intervals 3 -7 (minutes 6 - 21) for each newt (mean \pm SE).

A. Statistics

One-way ANOVA

$F(4, 75) = 1.23; p < 0.304$

B.



C.

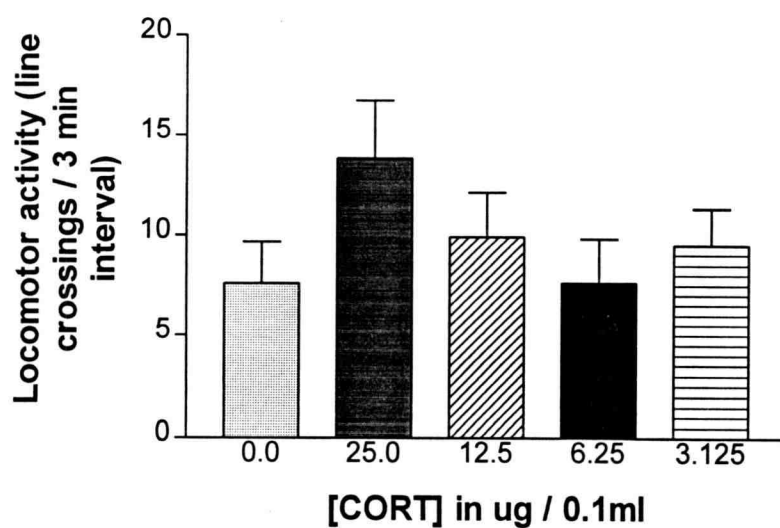


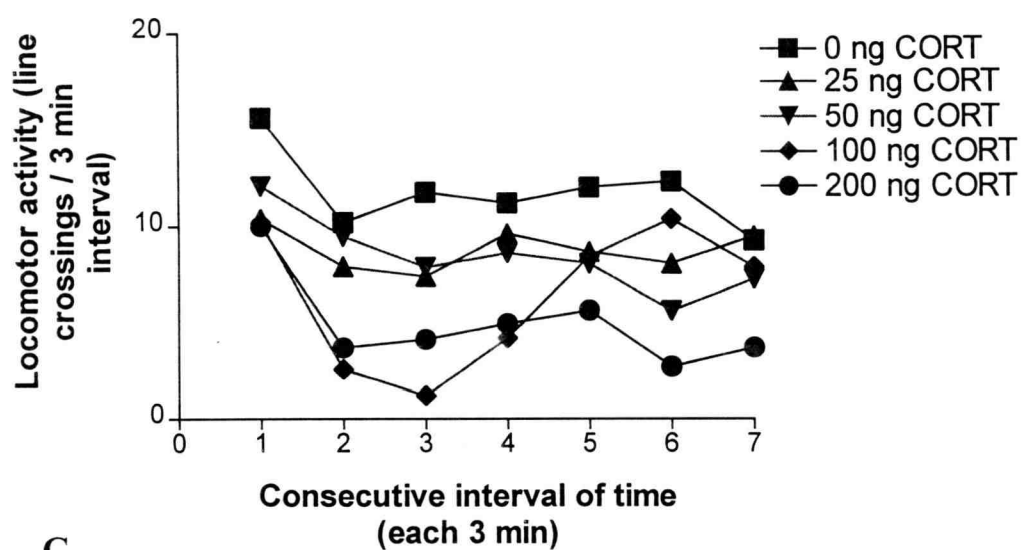
Fig. B.3 Effects of Fluoxetine (icv) and CORT (ip) on locomotor activity in male roughskin newts. **A.** Statistical analysis and results. **B.** Time course showing number of line crossings for the 21 minutes immediately following the icv injection divided into 7 consecutive 3-minute intervals. **C.** Mean locomotor activity per 3 minute interval calculated as the mean of the average of intervals 3 -7 (minutes 6 - 21) for each newt (mean \pm SE).

A. Statistics

One-way ANOVA

$$F(4, 75) = 1.39; p < 0.247$$

B.



C.

